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Short communication

Identification and validation of internal reference genes for real-time quantitative polymerase chain reaction-based studies in *Hyalomma anatolicum* ticks

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ABSTRACT

Crimean-Congo hemorrhagic fever (CCHF) is an emerging tick-borne viral disease caused by the orthonairovirus CCHF virus (CCHFV). Ticks of the genus *Hyalomma* are the viral reservoir and they represent the main vector transmitting the virus to their hosts during blood feeding. However, how CCHFV replicates in its natural arthropod host cells and the nature of virus/host interactions are still largely unknown. With the aim of developing tools for use in this field, we identified and validated expression of four candidate endogenous control tick genes in a *Hyalomma anatolicum*-derived cell line. These genes will be useful for normalization of viral/cellular transcripts in infection/expression studies or as internal controls in molecular epidemiology surveys of pathogens transmitted by *Hyalomma* ticks.

1. Introduction

Ticks of the genus *Hyalomma* are the main vectors and the natural reservoir of the orthonairovirus Crimean-Congo hemorrhagic fever virus (CCHFV) which is the most important emerging tick-borne virus affecting humans in Asia, Africa and Europe (Spengler et al., 2018). They are also major vectors of protozoan parasites of the genus *Theileria* affecting domestic ruminants (Jongejan and Uilenberg, 2004).

CCHFV usually circulates between asymptomatic hosts (wild and domestic mammals and ticks) in an enzootic cycle while human infection represents an accidental event. CCHFV persistently infects *Hyalomma ticks,* in which it shows transstadial survival and a transovarial transmission whereas mammalian infection is associated with a transitory viremia (Gargili et al., 2017). Human infections are largely asymptomatic; however in some people infection results in nonspecific febrile symptoms, which can progress to a serious hemorrhagic syndrome with a high case fatality rate (Akıncı et al., 2013; Bente et al., 2013). Normally, people who visit rural areas or people who are in contact with wildlife are more exposed to CCHFV infection, as well as people belonging to categories of workers sharing the same environment as the tick which serves as the natural host of CCHFV (Akıncı et al., 2013; Bente et al., 2013).

Despite the rapid increase in knowledge of CCHFV biology and the development of diagnostic tools in the last decade, there is still a large gap in the characterization of virus/vector interactions. This topic represents one of the main goals of current CCHFV research to increase the knowledge of virus biology, and it could suggest new strategies for control of virus spread (Papa et al., 2015). Recently, we have developed a CCHFV infection model based on *Hyalomma anatolicum* embryo-derived cell lines providing the opportunity to study viral-vector interaction in an *in vitro* model that is easier to handle than live ticks (Bell-Sakyi et al., 2012; Salata et al., 2018). However, the genome of *H. anatolicum* has not yet been sequenced, and this limits the possibilities for studying viral replication and modulation of cellular gene expression after infection. Host cell housekeeping genes are frequently used to normalize the levels of RNA expression between different samples but until now, no published data are available for ticks of the genus *Hyalomma*. Here we report the identification of endogenous reference genes

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that can be useful as tools to investigate CCHFV/tick interactions as well as for molecular epidemiology studies involving *Hyalomma* ticks.

2. Materials and methods

2.1. Cell culture

All culture media and supplements were obtained from Gibco unless otherwise indicated. *H. anatolicum* HAE/CTVM9 cells (Bell-Sakyi, 1991) were grown in L-15/MEM medium [equal volumes of L-15 (Leibovitz) and Minimal Essential Medium with Hank's salts supplemented with 10 % tryptose phosphate broth, 20 % fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin], and incubated in sealed flat-sided culture tubes (Nunc, Thermo-Fisher Scientific) in ambient air at 32 °C.

2.2. Illumina sequencing and assembly of the tick RNA data

HAE/CTVM9 cells were harvested, spun down at 335 rcf for 10 min at 4 °C, and washed once with PBS before lysis with TRIzol LS reagent (Invitrogen). Total RNA was purified from the aqueous phase using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Then, total RNA was subjected to sample preparation with the TrueSeq Stranded mRNA HT sample prep kit (Illumina Inc.). The sequencing was carried out with a 500 cycle MiSeq Reagent kit v2 (Illumina Inc.). Gene transcripts were assembled with the Trinity software suite that enables RNA expression analysis of RNA-Seq data in absence of a reference genome (Haas et al., 2013).

2.3. RNA isolation and qRT-PCR analysis

Total RNA extraction from HAE/CTVM9 cells was performed as described above. Primers and probes were developed using the primer design module of the CLC Genomics Workbench 7.5 (CLCBio, Aarhus) and were used at final concentrations of 900 nM and 250 nM respectively. Real-time RT-PCR amplification was done in duplicate for each sample using the Superscript III Platinum One-step kit (Invitrogen) in a Roche LightCycler 4.0 Instrument under the following conditions: reverse transcription, 10 min at 50 °C; denaturation, 2 min at 95 °C; amplification 45 cycles of 10 s at 95 °C for the denaturation and 40 s at 60 °C for the annealing/extension step. RNA expression levels (Ct) were used to calculate ΔCt for pairs of genes as previously described (Pecere et al., 2013; Silver et al., 2006).

3. Results

3.1. Identification of housekeeping gene candidates for RNA-expression studies in Hyalomma-derived tick cell lines

In order to identify housekeeping genes expressed by HAE/CTVM9 tick cells, a preliminary RNA-Seq experiment was carried out on an Illumina MiSeq instrument obtaining roughly one million reads that allowed the assembly of ~200,000 transcripts. We selected four candidate genes [β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), eukaryotic translation elongation factor 1 alpha 1 (EF1A) and ribosomal protein L13A] that belong to different cellular pathways, which significantly reduces the chance that genes might be co-regulated, and for which long highly-homologous contigs were obtained. Furthermore, these genes have been already found to be stably expressed in ixodid ticks of different genera (Nijhof et al., 2009; Browning et al., 2012; Koci et al., 2013). The Trinity-derived contigs were used as the template for TaqMan real time PCR system primer design. Primer length was set to 18–35 nucleotides, GC content to 0.4 − 0.6 and melting temperature to 58 − 62 °C. The primers and probes designed for the study are presented in Table 1. The expression of the selected genes identified by the transcriptomic analysis was confirmed in HAE/CTVM9 cells by real-time RT-PCR amplification from new RNA samples extracted from HAE/CTVM9 cells using the designated primers and probe systems. No amplification signals were obtained using RNAs from Vero or SW13 mammalian cells (data not shown).

Table 1
Primers and probes used to amplify fragments of *Hyalomma anatolicum* housekeeping genes for RNA expression normalization by qRT-PCR.

Tick gene	Oligo	Sequence – 5′ to 3′	Size (bp)
EF1A	EF1A-F	GGGAGGTGAATAGTTAGGGT	161
	EF1A-R	GCGCCAGTCATCTAAGGAA	
	EF1A-P	FAM-TGTGAGTGGCAGAGAATGTGGTGGGT-TAMRA	
ACTB	ACTB-F	TGCGGCTTCTTGTCTGTT	271
	ACTB-R	ACCCATACCCACCATGAC	
	ACTB-P	FAM-CCGCTTCCCCGACCCCATCAAAAAC-TAMRA	
GAPDH	GAPDH-F	CCGAAGTTGTCGTGGATG	120
	GAPDH-R	AATGTTTGTGATGGGCGT	
	GAPDH-P	FAM- ATGCTCGGATTGTAGGTGGTGTGGT-TAMRA	
L13A	L13A-F	ACTTCCAGCCAACCTCAT	235
	L13A-R	TACCACTTCAGGGCACCCA	
	L13A-P	FAM-TGCCTCCACCATACCACCGCAT-TAMRA	

Primers and probes were designed using the software CLC Genomics Workbench applied to sequences obtained from RNA-seq analysis of *H. anatolicum* HAE/CTVM9 cells by Illumina MiSeq System (Illumina Inc.).

3.2. Comparative evaluation of the identified housekeeping genes

To validate the candidate housekeeping genes, we used the ΔCt approach that evaluates the relative RNA expression stability between candidate transcripts (Silver et al., 2006). The ΔCt approach compared the relative expression of "pairs of genes" within each sample tested. To analyze the stability of expression of the candidate genes in HAE/CTVM9 cells, samples of actively growing cells were collected at 24 h intervals over a seven-day period and total RNA was extracted for real-time RT-PCR analysis to determine the Ct value. If the ΔCt value between two genes remains similar for all the RNA samples tested, it means that the expression of the two genes analyzed is stable. If the ΔCt is variable, it means that there is a modulation of the gene expression and the tested gene is not suitable as an endogenous reference gene. Moreover, the standard deviation of the ΔCt can represent a measure to evaluate the stability of the gene expression, thereby obtaining an order of rank for the different genes. The overall ranking of the candidate genes, based on the standard deviation of ΔCt that represented the variability of relative expression between the selected genes, was: EF1A and ACTB, followed by GAPDH, and lastly ribosomal protein L13A (Table 2). All four selected endogenous *H. anatolicum* genes showed a low level of variation (standard deviation ≤ 0.31), indicating that they are suitable for RNA normalization purposes.

4. Discussion

CCHFV is the most important and widespread tick-borne hemorrhagic fever virus worldwide. During the last two decades, many outbreaks have been reported, as well as an expansion of its range in Africa, Europe, the Middle East and Asia (Spengler et al., 2018). Considering its potential impact on public heath, in 2018 the World Health Organization included CCHF in the WHO R&D Blueprint list that indicated the priority diseases for which more effort should be made to develop control strategies (Mehand et al., 2018).

To date no specific antivirals or vaccines have been approved for CCHFV and the best option is prevention, mainly by controlling or avoiding the vectors. Unfortunately, CCHFV can be handled only in

Table 2
Comparison of candidate *Hyalomma anatolicum* housekeeping genes.

Gene comparison	Mean ΔCt	Std Dev	Mean Std Dev
EF1A vs ACTB	2.49	0.16	
EF1A vs GAPDH	3.62	0.21	
EF1A vs L13A	1.23	0.25	0.21
ACTB vs EF1	2.49	0.16	
ACTB vs GAPDH	1.13	0.17	
ACTB vs L13A	1.26	0.29	0.21
GAPDH vs EF1A	3.62	0.21	
GAPDH vs ACTB	1.13	0.17	
GAPDH vs L13A	2.39	0.31	0.23
L13A vs EF1A	1.23	0.25	
L13A vs ACTB	1.26	0.29	
L13A vs GAPDH	2.39	0.31	0.28

Mean ΔCt values indicate the mean difference in RNA expression levels in *H. anatolicum* cells between the selected genes tested in two independent experiments. Standard deviation (Std Dev) is given for the variation in Ct values over the samples tested.

high-containment, biosafety level (BSL)-4 laboratories, limiting the scope of research activities. In fact, very little information is available about the biology of CCHFV in its natural reservoir. Although several *Hyalomma* spp. tick cell lines are now available (Bell-Sakyi et al., 2018), only two studies have been published using these interesting models to propagate CCHFV (Bell-Sakyi et al., 2012; Salata et al., 2018). An additional problem is the lack of specific tools to study viral biology in ticks.

To throw light on this topic, we initiated an approach to study the transcriptomics of *Hyalomma* ticks and tick cell lines, but the lack of a sequenced genome made the analysis very difficult. However, the contigs obtained from this analysis were useful to select endogenous reference genes that are essential to normalize data in gene expression experiments or to evaluate replication and gene expression of CCHFV in tick cells. Amongst the best contigs available, we selected four genes that were previously shown to be constitutively expressed in other tick genera, thus representing promising candidates for reference genes (Nijhof et al., 2009; Browning et al., 2012; Koci et al., 2013). In agreement with the data in the literature, the ΔCt approach showed that in *H. anatolicum* tick cells the selected genes presented a constant level of expression. Indeed, they can be used as good reference genes for the normalization of gene expression. In a previous study, we used the EF1A gene to normalize the values obtained for the yield of CCHFV RNA after infection of two *H. anatolicum*-derived tick cell lines, and the results obtained were consistent with the viral titer and the expression of viral nucleoprotein (Salata et al., 2018). Overall, our results support the suitability in particular of ACTB and EF1A as endogenous reference genes for this tick species. However, further studies with cell lines derived from additional *Hyalomma* species, such as *Hyalomma dromedarii* and *Hyalomma lusitanicum* (Bell-Sakyi et al., 2018), and *Hyalomma* ticks at different stages of the life cycle, are needed to confirm broader applicability of these genes across the genus.

In conclusion, in this study we identified four different *H. anatolicum* reference genes and developed a real-time RT-PCR protocol for them that will be useful for normalization of viral/cellular transcripts in infection/expression studies or as an internal control in molecular epidemiology surveys of pathogens transmitted by *Hyalomma* ticks.

Author contributions

CS, VM and ML performed the experimental procedures and data collection. CS and AM designed the experimental procedures. LBS generated and supplied the HAE/CTVM9 cells and the cell culture protocols. CS drafted the manuscript. VM, ML, LBS and AM revised the manuscript.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2020.101417.

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